One-Dimensional Electrophoresis Using Nondenaturing Conditions

Nondenaturing or "native" electrophoresis—i.e., electrophoresis in the absence of denaturants such as detergents and urea—is an often-overlooked technique for determining the native size, subunit structure, and optimal separation of a protein. Because mobility depends on the size, shape, and intrinsic charge of the protein, nondenaturing electrophoresis provides a set of separation parameters distinctly different from mainly size-dependent denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; *UNITS 10.2A*) and charge-dependent isoelectric focusing (IEF; *UNITS 10.3 & 10.4*). Two protocols are presented below. Continuous PAGE (see Basic Protocol) is highly flexible, permitting cationic and anionic electrophoresis over a full range of pH. The discontinuous procedure (see Alternate Protocol) is limited to proteins negatively charged at neutral pH but provides high resolution for accurate size calibration.

CONTINUOUS ELECTROPHORESIS IN NONDENATURING POLYACRYLAMIDE GELS

BASIC PROTOCOL

Separation of proteins by nondenaturing electrophoresis requires the same type of equipment used for denaturing slab gels (*UNIT 10.2A*) and is adaptable to a range of gel sizes (e.g., from 7.3 × 8.3–cm minigels to 14 × 16–cm full-size gels) and matrix types (e.g., single-concentration and gradient gels). This protocol outlines straightforward procedures for making acrylamide solutions, casting separating gels (stacking gels are omitted), loading samples, and conducting electrophoresis. Continuous systems, although flexible, do not give the high-resolution separation found in discontinuous systems (see Alternate Protocol).

Separation in a continuous system (i.e., in which the same buffer is used for preparing acrylamide solutions and filling electrophoresis chambers) is governed by pH, and this protocol describes four types of buffers useful over discrete ranges from pH 3.7 to pH 10.6. Use of unadjusted acetic acid gel buffer can extend the range to pH 2.0. The choice of pH and thus the buffer system depends on the protein being studied (i.e., its isoelectric point) and often must be determined empirically. In general, the system should be between pH 5.0 and 8.0 for optimal results. Extremes of pH can lead to precipitation or denaturation of the protein. Acrylamide concentrations are empirically determined, but the higher the percent acrylamide, the sharper the protein bands.

It is important to include native protein standards in the electrophoresis runs. Several manufacturers supply standards for isoelectric focusing that are also suitable for native electrophoresis. The standards have a range of isoelectric points and will carry a net positive, negative, or zero charge depending on the pH of the gel system. Alternatively, Sigma supplies a standard kit that is useful for calculating molecular weights under neutral pH, nondenaturing conditions. The samples and standard proteins, should be used at concentrations of ~1 to 2 μg/μl.

Materials

4× acetic acid gel buffer (200 mM acetic acid, pH 3.7 to 5.6; see recipe)
4× phosphate gel buffer (400 mM sodium phosphate, pH 5.8 to 8.0; see recipe)
4× Tris gel buffer (200 mM Tris·Cl, pH 7.1 to 8.9; see recipe)
4× glycine gel buffer (200 mM glycine, pH 8.6 to 10.6; see recipe)
300 mM sodium sulfite (0.38 g in 10 ml H_2O; used in acetic acid gel preparation)
Protein samples to be analyzed

Contributed by Sean R. Gallagher
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Native protein standards
Electrophoresis buffer: appropriate 4× gel buffer diluted to 1× with H_2O
75-ml side-arm flask (used in gel preparation)

Additional reagents and equipment for gel electrophoresis (*UNIT 10.2A*) and staining proteins in gels (*UNIT 10.6*)

Prepare the gel

1. Assemble the glass-plate sandwich of the gel electrophoresis unit and secure it to the casting stand.

   Either single-concentration or gradient gels can be used in the minigel or standard-size format. Gradient gels will enhance the band sharpness of the separated proteins.

2. Prepare acrylamide solutions according to the recipes in Table 10.2B.1, Table 10.2B.2, Table 10.2B.3, or Table 10.2B.4, adding the ammonium persulfate and TEMED just before use.

   Deaeration of the solution before the polymerization catalysts are added will speed polymerization by removing inhibitory oxygen, but is not generally required. The pH used depends on many factors. The most important are the pI values of both the protein of interest and any contaminants, as well as protein mobility and protein solubility. Determining which pH and thus which buffer system to use is largely empirical. However, extremes of pH (<4.0 and >9.0) can lead to denaturation and should be avoided. Prior knowledge of the pI of a protein (UNITS 10.3 & 10.4) allows determination of the net charge under the

Table 10.2B.1 Recipes for Acetic Acid Nondenaturing Polyacrylamide Gels[a]: pH range 3.7 to 5.6[b]

Stock solution[c]	Final acrylamide concentration in gel (%)[d]						
	5	7.5	10	12.5	15	17.5	20
30% acrylamide/0.8% bisacrylamide	6.7	10	13.3	16.8	20	23.32	26.6
300 mM sodium sulfite[e]	0.4	0.4	0.4	0.4	0.4	0.4	0.4
4× acetic acid gel buffer	10	10	10	10	10	10	10
H_2O	22.58	19.28	15.98	12.48	9.28	5.96	2.68
10% (w/v) ammonium persulfate[e]	0.3	0.3	0.3	0.3	0.3	0.3	0.3
TEMED[f]	0.02	0.02	0.02	0.02	0.02	0.02	0.02

Preparation of gel

In a 75-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution (see Table 10.2A.1), 300 mM sodium sulfite, 4× acetic acid gel buffer (see Reagents and Solutions), and H_2O. If desired to speed polymerization, degas under vacuum ~5 min. Add 10% ammonium persulfate and TEMED. Swirl gently to mix. Use immediately.

[a]The recipes produce 40 ml gel solution, which is adequate for one gel of dimensions 1.5 mm × 14 cm × 16 cm or two gels of dimensions 0.75 mm × 14 cm × 16 cm.

[b]The pH range can be extended to ~2.0 (the pH of acetic acid) by using unadjusted acetic acid in place of 4× acetic acid gel buffer, although there is little buffering capacity at this pH.

[c]All reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.

[d]Units of numbers in table body are milliliters. The desired percentage of acrylamide in the gel solution depends on the molecular size of the protein being separated.

[e]Must be freshly made. Sodium sulfite is needed for efficient polymerization at acid pH.

[f]Added just before polymerization.

Table 10.2B.2 Recipes for Phosphate Nondenaturing Polyacrylamide Gels[a]: pH range 5.8 to 8.0

Stock solution[b]	Final acrylamide concentration in gel (%)[c]						
	5	7.5	10	12.5	15	17.5	20
30% acrylamide/0.8% bisacrylamide	6.7	10	13.3	16.8	20	23.32	26.6
4× phosphate gel buffer	10	10	10	10	10	10	10
H_2O	23.08	19.78	16.48	12.98	9.78	6.46	3.18
10% (w/v) ammonium persulfate[d]	0.2	0.2	0.2	0.2	0.2	0.2	0.2
TEMED[e]	0.02	0.02	0.02	0.02	0.02	0.02	0.02

Preparation of gel

In a 75-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution (see Table 10.2A.1), 4× phosphate gel buffer (see Reagents and Solutions), and H_2O. If desired, degas under vacuum ~5 min to speed polymerization. Add 10% ammonium persulfate and TEMED. Swirl gently to mix. Use immediately.

[a]The recipes produce 40 ml gel solution, which is adequate for one gel of dimensions 1.5 mm × 14 cm × 16 cm or two gels of dimensions 0.75 mm × 14 cm × 16 cm.
[b]All reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.
[c]Units of numbers in table body are milliliters. The desired percentage of acrylamide in the gel solution depends on the molecular size of the protein being separated.
[d]Must be freshly made.
[e]Added just before polymerization.

separation conditions (i.e., if gel pH < protein pI, the protein will have a net positive charge; if gel pH > protein pI, the protein will be negatively charged).

3. Pour gel to 2 cm from the top of the gel mold and insert the comb. Avoid trapping air bubbles under the comb teeth.

   Air bubbles will cause small semicircular depressions in the well and lead to distortions in the protein banding pattern.

4. Allow gel solution to polymerize 1 to 2 hr.

   Polymerization is indicated by a sharp optical discontinuity around the wells.

Prepare samples and load the wells

5. Solubilize the protein sample to be analyzed using 5% (w/v) sucrose in water or dilute (1 to 5 mM) gel buffer if possible. Also prepare native protein standards.

   The concentration of protein will vary depending on the sample complexity and detection method. For Coomassie blue staining of highly enriched samples such as the standards, use 1 to 2 mg/ml (1 to 2 μg/μl). For more complex mixtures, use 5 to 10 mg/ml (5 to 10 μg/μl). Load 10- to 100-fold less for silver staining. In general, samples should be loaded in a minimum volume, preferably 10 to 20 μl for 0.75- and 1.5-mm-thick gels, respectively. With thin gels, this means using a more concentrated protein sample.

6. Remove comb carefully and rinse wells with electrophoresis buffer (appropriate 4× gel buffer diluted to 1×).

   Rinsing with electrophoresis buffer is needed to remove residual unpolymerized acrylamide monomer, which will continue to polymerize after comb removal, creating uneven wells that may interfere with sample loading.

Table 10.2B.3 Recipes for Tris Nondenaturing Polyacrylamide Gels[a]: pH range 7.1 to 8.9

Stock solution[b]	Final acrylamide concentration in gel (%)[c]						
	5	7.5	10	12.5	15	17.5	20
30% acrylamide/0.8% bisacrylamide	6.7	10	13.3	16.8	20	23.32	26.6
4× Tris gel buffer	10	10	10	10	10	10	10
H_2O	23.08	19.78	16.48	12.98	9.78	6.46	3.18
10% (w/v) ammonium persulfate[d]	0.2	0.2	0.2	0.2	0.2	0.2	0.2
TEMED[e]	0.02	0.02	0.02	0.02	0.02	0.02	0.02

Preparation of gel

In a 75-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution (see Table 10.2A.1), 4× Tris gel buffer (see Reagents and Solutions), and H_2O. If desired, degas under vacuum ~5 min to speed polymerization. Add 10% ammonium persulfate and TEMED. Swirl gently to mix. Use immediately.

[a]The recipes produce 40 ml gel solution, which is adequate for one gel of dimensions 1.5 mm × 14 cm × 16 cm or two gels of dimensions 0.75 mm × 14 cm × 16 cm.

[b]All reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.

[c]Units of numbers in table body are milliliters. The desired percentage of acrylamide in the gel solution depends on the molecular size of the protein being separated.

[d]Must be freshly made.

[e]Added just before polymerization.

7. Fill wells with electrophoresis buffer. If desired, prerun gel.

The gel can be prerun at this point to remove any charged material such as ammonium persulfate from the gel prior to loading the sample. Assemble the electrophoresis unit and fill the buffer chambers with electrophoresis buffer. Run the gel at 300 V until the current no longer drops. This should take ~30 min. Disassemble the unit, discard the buffer, and proceed to the next step.

8. Carefully load up to 10 µl (0.75-mm gels) or 20 µl (1.5-mm gels) sample per lane as a thin layer at the bottom of the wells. Load control wells with native protein standards. Add an equal volume of electrophoresis buffer to any empty wells to prevent spreading of adjoining lanes.

Mobility (R_f) markers require special consideration in nondenaturing gel systems. For cationic systems, cytochrome c (pI ~9 to 10, 5 to 10 µg/lane) works well as an R_f marker. Bromphenol blue (10 µg/ml) is a suitable marker for anionic systems. The marker should be included in the solubilization buffer with the sample.

Perform the separation

9. Assemble the gel unit, fill the upper and lower buffer chambers with electrophoresis buffer, and connect the unit to the power supply. Set current to 30 mA for a 1.5-mm-thick gel (15 mA for a 0.75-mm-thick gel).

If the protein is negatively charged under the separation conditions, then the standard SDS-PAGE electrode polarity should be used (proteins will migrate to the anode or positive electrode; see UNIT 10.2A). If the protein is positively charged, then the electrodes should be reversed at the power supply (i.e., red high-voltage cable to the black output and black high-voltage lead to the red output) so the positively charged protein migrates to the negative cathode.

Table 10.2B.4 Recipes for Glycine Nondenaturing Polyacrylamide Gels[a]: pH range 8.6 to 10.6

Stock solution[b]	Final acrylamide concentration in gel (%)[c]						
	5	7.5	10	12.5	15	17.5	20
30% acrylamide/0.8% bisacrylamide	6.7	10	13.3	16.8	20	23.32	26.6
4× glycine gel buffer	10	10	10	10	10	10	10
H_2O	23.08	19.78	16.48	12.98	9.78	6.46	3.18
10% (w/v) ammonium persulfate[d]	0.2	0.2	0.2	0.2	0.2	0.2	0.2
TEMED[e]	0.02	0.02	0.02	0.02	0.02	0.02	0.02

Preparation of gel

In a 75-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution (see Table 10.2A.1), 4× glycine gel buffer (see Reagents and Solutions), and H_2O. If desired, degas under vacuum ~5 min to speed polymerization. Add 10% ammonium persulfate and TEMED. Swirl gently to mix. Use immediately.

[a]The recipes produce 40 ml gel solution, which is adequate for one gel of dimensions 1.5 mm × 14 cm × 16 cm or two gels of dimensions 0.75 mm × 14 cm × 16 cm.

[b]All reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.

[c]Units of numbers in table body are milliliters. The desired percentage of acrylamide in the gel solution depends on the molecular size of the protein being separated.

[d]Must be freshly made.

[e]Added just before polymerization.

10. Continue electrophoresis until the R_f marker reaches the bottom of the gel.

For minigels, electrophoresis will take 1 to 2 hr. Standard gels require 4 to 6 hr runs.

11. Turn off power supply, disassemble the unit, and remove gel from sandwich.

12. Stain the gel according to *UNIT 10.6*.

NATIVE DISCONTINUOUS ELECTROPHORESIS AND GENERATION OF MOLECULAR WEIGHT STANDARD CURVES (FERGUSON PLOTS)

ALTERNATE PROTOCOL

One straightforward approach to discontinuous native electrophoresis is to leave out the SDS and reducing agent (DTT) from the standard Laemmli SDS-PAGE protocol (*UNIT 10.2A*). The gels are prepared as described in *UNIT 10.2A* except that the sample buffer contains no SDS or DTT (samples are not heated), and the gel and electrophoresis solutions are prepared without SDS. This protocol illustrates the separation of standard proteins at four different concentrations of acrylamide and how the results are used to construct a molecular weight standard curve (Ferguson plot) without the need for SDS. By plotting relative mobility against %T (percentage weight per volume of acrylamide plus bisacrylamide in the gel), the presence of isoforms and multimeric proteins can also be detected.

Materials

4× Tris·Cl, pH 8.8 (1.5 M Tris·Cl; *APPENDIX 2*)
4× Tris·Cl, pH 6.8 (0.5 M Tris·Cl; *APPENDIX 2*)
Protein sample of interest
2× Tris/glycerol sample buffer (see recipe)

Native protein standards (e.g., Sigma nondenatured protein molecular weight kit)
Tris/glycine electrophoresis buffer (see recipe)

1. Assemble the glass-plate sandwich of the gel electrophoresis unit and place it in the casting stand.

2. Prepare and cast the gels, using 4× Tris·Cl, pH 8.8, for the separating gel and 4× Tris·Cl, pH 6.8, for the stacking gel instead of the SDS-containing counterparts (Table 10.2A.1). Prepare a minimum of four separate gels at different acrylamide concentrations.

   A typical range of concentrations is from 5% to 12.5% (e.g., 5%, 7.5%, 10%, 12.5% acrylamide). As with SDS-PAGE, typical gel thickness ranges from 0.75 to 1.5 mm. The 0.75-mm-thick gels are recommended because they offer a combination of fast staining and high resolution.

3. Mix protein sample of interest 1:1 with 2× Tris/glycerol sample buffer to attain a 1 to 2 μg/μl final concentration. Also prepare native protein standards. Remove comb, rinse wells, and load 10 to 20 μl per well for Coomassie brilliant blue staining, and 1 to 2 μl for silver staining.

   Some proteins must be dissolved in 50 mM NaCl or water to become fully solubilized prior to mixing with the sample buffer (Sigma, 1986).

4. Assemble gel electrophoresis unit, using Tris/glycine electrophoresis buffer to fill both lower and upper buffer chambers. Connect power supply and conduct electrophoresis.

   Conditions for separation are the same as for discontinuous SDS-PAGE (i.e., 30 mA for 1.5-mm-thick gels, 15 mA for 0.75-mm-thick gels). For standard-size gels the separation takes 4 to 5 hr; for minigels, 1 to 2 hr is required. Alternatively, standard gels can be run at 4 to 6 mA/gel overnight.

5. After the bromphenol blue R_f marker has reached the bottom of the gel, fix and stain the proteins in the gels according to *UNIT 10.6*. Estimate relative mobilities of the proteins.

   An example of a stained gel is shown in Figure 10.2B.1. A minimum of four gel concentrations is recommended. In Figure 10.2B.1, Sigma native molecular weight standards were separated on 5%, 7.5%, 10%, and 12.5% acrylamide gels (5.1%, 7.7%, 10.3%, and 12.8% T, respectively).

6. Plot log R_f against gel concentration (% T) (Fig. 10.2B.2). Determine the slope of K_r using linear regression.

7. Plot $-\log K_r$ of the curves from step 6 against log molecular weight of the standards (Fig. 10.2B.3). Determine the slope using linear regression.

8. Estimate the size of the standards and unknowns from the generated curve (Ferguson plot).

   Use the curve generated by linear regression to estimate the predicted size of the standard for comparison to the actual size stated by the supplier. This indicates the accuracy of the curve. The $-\log K<D_r$ value (y) of the unknown is then used to predict the molecular weight (x).

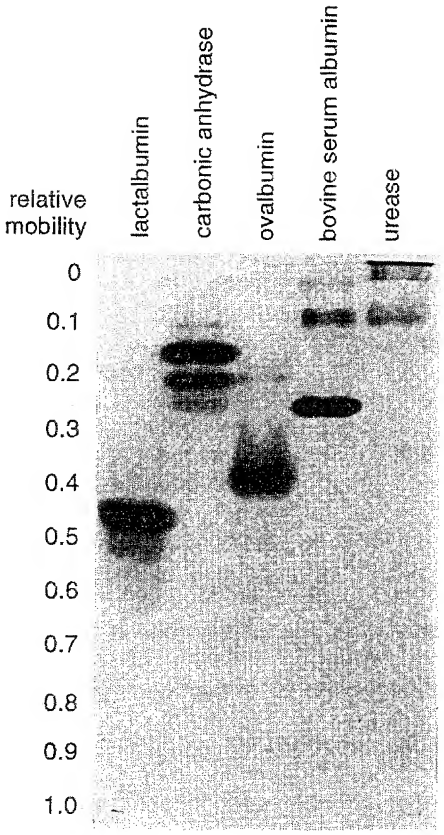

Figure 10.2B.1 Separation of native protein standards under nondenaturing conditions by discontinuous polyacrylamide gel electrophoresis at 12.8% T. Approximately 20 µg protein was loaded per lane on a 1.5-mm-thick, 16-cm-long gel. The gel and samples were prepared according to the Alternate Protocol and were electrophoresed 16 hr at 6 mA. Proteins were stained with Coomassie blue.

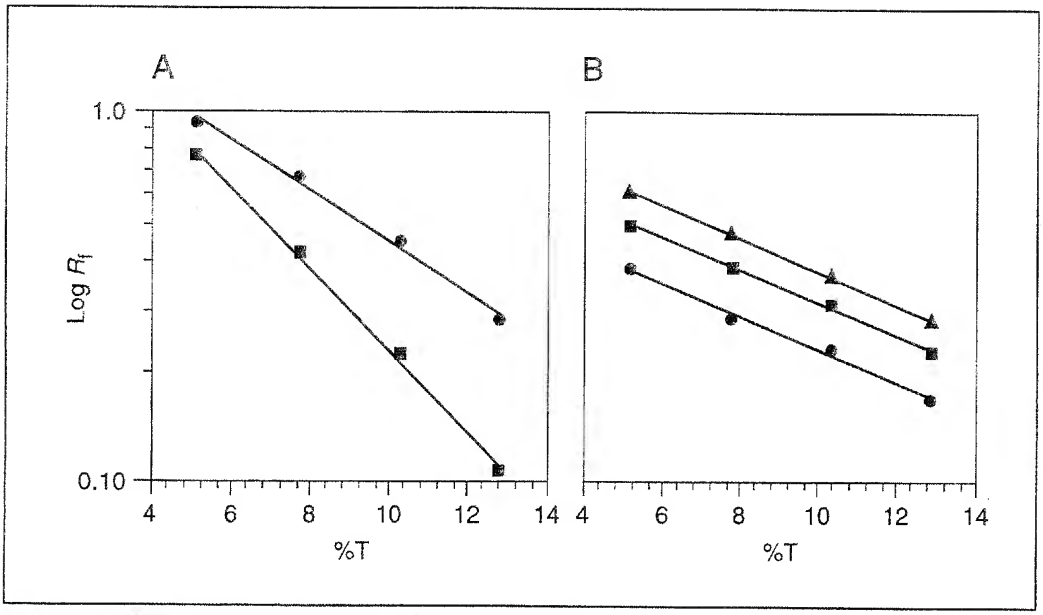

Figure 10.2B.2 Effect of %T on the relative mobility of several native proteins. The relative mobility (R_f) of the standard proteins shown in Figure 10.2B.1 was determined at four different gel concentrations and plotted as log R_f against %T. See text for details. (**A**) BSA monomer (squares) and dimer (circles); (**B**) carbonic anhydrase isoforms.

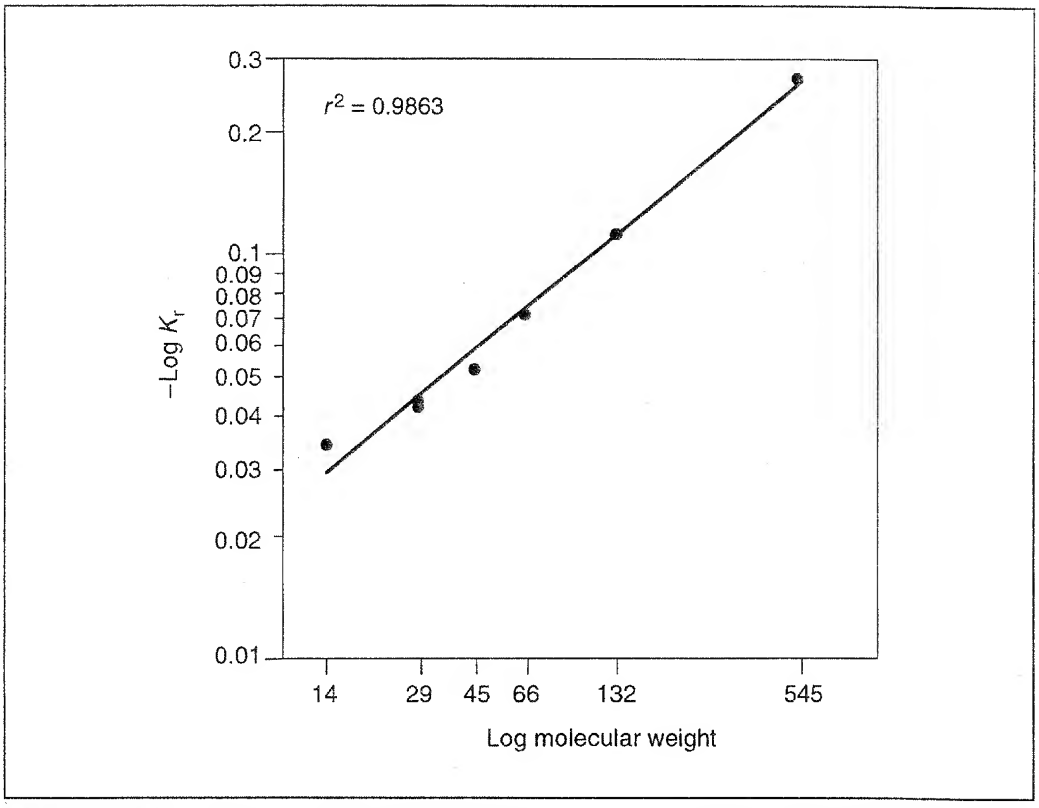

Figure 10.2B.3 Native molecular weight standard curve. The –log slope of the line (K_r) from Figure 10.2B.2 is plotted against log molecular weight of the standards.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent for the preparation of all buffers. For common stock solutions, see APPENDIX 2; for suppliers, see APPENDIX 4.

Acetic acid gel buffer, 4× (200 mM acetic acid, pH 3.7 to 5.6)

11.49 ml glacial acetic acid
Add to 500 ml H_2O
Adjust to pH 3.7 to 5.6 with 1 M NaOH
Add H_2O to 1000 ml
Store up to 1 month at 4°C

Glycine gel buffer, 4× (200 mM glycine, pH 8.6 to 10.6)

15.01 g glycine
Add to 500 ml H_2O
Adjust to pH 8.6 to 10.6 with 1 M NaOH
Add H_2O to 1000 ml
Store up to 1 month at 4°C

Phosphate gel buffer, 4× (400 mM sodium phosphate, pH 5.8 to 8.0)

55.2 g $NaH_2PO_4 \cdot H_2O$
Add to 500 ml H_2O
Adjust to pH 5.8 to 8.0 with 1 M NaOH
Add H_2O to 1000 ml
Store up to 1 month at 4°C

Tris gel buffer, 4× (200 mM Tris·Cl, pH 7.1 to 8.9)

24.23 g Tris base
Add to 500 ml H_2O
Adjust to pH 7.1 to 8.9 with 1 M HCl
Add H_2O to 1000 ml
Store up to 1 month at 4°C

2× Tris/glycerol sample buffer

25 ml 0.5 M Tris·Cl, pH 6.8
20 ml glycerol
1 mg bromphenol blue
Add H_2O to 100 ml and mix
Store in 1-ml aliquots up to 6 months at –70°C

Tris/glycine electrophoresis buffer

15.1 g Tris base
72.0 g glycine
H_2O to 5000 ml
Store up to 1 month at 4°C

COMMENTARY

Background Information

Under nondenaturing conditions, in which protein activity, native charge, and conformation are sustained, electrophoretic separation depends on many factors, including size, shape, and charge. Characteristics such as intrinsic molecular weight (i.e., in the absence of denaturation), the number of isoforms, and the presence of multimeric proteins can be determined with nondenaturing electrophoresis (often called native electrophoresis).

The most important application of nondenaturing electrophoresis is in the determination of native protein size (Alternate Protocol). Ferguson plots (reviewed by Andrews, 1986) were first described for starch gels (Ferguson, 1964) and then for polyacrylamide gels (Hedrick and Smith, 1968). Ferguson plots are prepared by separating proteins under nondenaturing conditions at several different gel concentrations. As the acrylamide concentration (%T) is increased, the relative mobility (R_f) of the protein decreases. This is plotted as log relative mobility (on the *y* axis) versus %T (on the *x* axis) to produce a straight line. The slope of this line is referred to as the retardation coefficient (K_r) and measures how effectively a protein is slowed by the increase in %T. Large proteins will be retarded much more significantly than small proteins with increasing gel concentration, with the size of the protein being proportional to the slope of the curve. Once the K_r plots for several size standards are generated (Fig. 10.2B.2), the K_r values are plotted against the molecular weight of the standard proteins using a log-log graph (Fig. 10.2B.3). The retardation coefficient also depends on a large number of other variables including temperature, pH, buffer type, ionic strength, and %C (percent bisacrylamide cross-linker). All these factors should be kept constant for a given experiment.

In addition to estimated size, other types of information are available from the Ferguson plots (Rodbard and Chrambach, 1971; Andrews, 1986). For example, if two components differ in size but have the same charge per unit size (e.g., for a multimeric protein with identical subunits), curves similar to those illustrated by BSA monomer and dimer (Fig. 10.2B.2A) will result. Note that when the curve is extrapolated back to 0% T, it is evident that the monomer and the dimer have similar free solution mobilities. Furthermore, as the acrylamide concentration is increased, the separation between the two also increases. However, if two proteins have similar sizes but different amounts of charge, the curves will be parallel on the log plot. This is illustrated by the carbonic anhydrase isoforms (Fig. 10.2B.2B). In this example, optimal separation of the isoforms occurs at the lower concentrations of acrylamide as this is a log plot.

Further applications of nondenaturing electrophoresis include preparative purification. The pH of the gel determines the net charge on the protein. Below its isoelectric point (pI) a protein will have a net positive charge, whereas above its pI it will have a net negative charge.

In general, most proteins will be positively charged at pH 2.0 to 4.0; above pH 8.0, most proteins will be negatively charged. As these general guidelines imply, the majority of proteins have isoelectric points between pH 4.0 and 8.0. There are, however, many exceptions. A protein with a highly acidic isoelectric point (e.g., pepsin, with a pI of 2.2) will remain negatively charged at a pH down to its pI. Although a full range of pH options are given, extremes of pH (<4.0 and >9.0) should be avoided, if possible, to minimize denaturation or inactivation. By picking an appropriate electrophoresis pH, it is possible to ensure that the protein of interest will be either positively or negatively charged so that it can be selectively run into the gel, excluding a large proportion of contaminants that have the opposite or no charge. Furthermore, the pH conditions determine the mobility and can be adjusted to ensure a difference in mobility between the protein of interest and contaminants.

Continuous gel systems (see Basic Protocol) offer the most flexibility in terms of separation design. The pH can be tailored so that a given protein has a net positive, neutral, or negative charge. Depending on the polarity of the gel, the protein can then be excluded from or electrophoresed into the gel. Discontinuous gels have a fixed pH and gel polarity. For the nondenaturing Laemmli gel presented in the Alternate Protocol, the proteins of interest should have an isoelectric point of ≤7.0 in order to be negatively charged so that they move into the gel. Other more basic and more acidic discontinuous gel systems can be found in Hames (1990) and Schägger (1994).

Critical Parameters

The success of a gel separation under nondenaturing conditions depends on many factors, and two of the most important are protein solubility and isoelectric point. The protein must be soluble at the pH and the ionic strength of the gel, and it must be charged at that pH in order to move into the gel. If the protein experiences a pH below its isoelectric point, then it will have a net positive charge and will move to the negative electrode. Note that this is the reverse of typical SDS-PAGE. If the protein experiences a pH above its isoelectric point, it will have a net negative charge and will migrate to the positive electrode.

Solubility is a complex issue. Membrane-associated and other hydrophobic proteins are difficult to separate by nondenaturing electrophoresis (Schägger, 1994). Nonionic detergents at concentrations up to 1% and solubilizing reagents such as urea (4 to 8 M) can be used, but these reagents, especially urea, are likely to alter the protein's conformation and most likely the isoelectric point by exposing previously hidden charged groups. If detergent or urea must be included for solubilization, the minimum required to solubilize the protein should be used. Schägger (1994) lists several nonionic detergents suitable for solubilization. Among the more popular are octylglucoside and CHAPS. In general, detergents should be used near the critical micelle concentration (CMC; 0.001% to 1%, depending on the detergent).

The gel concentration has a dramatic effect on resolution and should be optimized in order to achieve the best separation and band sharpness. In general, increasing the %T will improve band sharpness.

Troubleshooting

Gel polymerization at acid pH can be problematic, and sodium sulfite is needed for efficient polymerization (Andrews, 1986). Both the ammonium persulfate and the sodium sulfite must be freshly made, and the highest quality reagents available should be used. Furthermore, the gel solutions should be at room temperature for effective polymerization.

If the protein does not enter the gel and no stained material is present at the well surface, try reversing the polarity of the electrode. If material concentrates at the top of the gel, try lowering the acrylamide concentration. Stained material at the top of the gel may also indicate poor solubilization, and increasing the ionic strength of the solubilization buffer or adding a small amount of urea and/or nonionic detergent may be required.

Anticipated Results

Proteins will resolve depending on their solubility and native charge at the chosen pH. Ideally, a distinct band representing the protein of interest will be visible. If the band is diffuse, then increasing the gel concentration or using a gradient gel will improve resolution. If the band is not visible, then the protein may be at its isoelectric point or may have moved out of the gel because it had the wrong charge. Continuous gel systems, although more versatile, will give lower resolution than discontinuous gels. Detergents or other solubilizing agents such as urea may be needed to fully solubilize and resolve the protein. Once the conditions that resolve the protein are determined, Ferguson plots will give indications of multiple

subunit structure, native size, and potential isoform relationships.

Time Considerations

Separations will be complete when the tracking dye or protein reaches the bottom of the gel. For minigels, this generally takes 1 to 2 hr, using 15 or 30 mA for 0.75- or 1.5-mm-thick gels, respectively. Standard-format gels require 4 to 5 hr at 15 or 30 mA for 0.75- or 1.5-mm-thick gels, respectively. Standard-format gels can also run overnight at 4 to 6 or 8 to 12 mA for 0.75- or 1.5-mm-thick gels, respectively.

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Key Reference

Andrews, 1986. See above.

Covers a variety of electrophoretic techniques, including nondenaturing electrophoresis and Ferguson plots.

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